A comparative study of class-D β-lactamases

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Three class-D β-lactamases (OXA2, OXA1 and PSE2) were produced and purified to protein homogeneity. 6β-Iodopenicillanate inactivated the OXA2 enzyme without detectable turnover. Labelling of the same β-lactamase with 6β-iodo[^{3}H]penicillanate allowed the identification of Ser-70 as the active-site serine residue. In agreement with previous reports, the apparent M_r of the OXA2 enzyme as determined by molecular-sieve filtration, was significantly higher than that deduced from the gene sequence, but this was not due to an equilibrium between a monomer and a dimer. The heterogeneity of the OXA2 β-lactamase on ion-exchange chromatography contrasted with the similarity of the catalytic properties of the various forms. A first overview of the enzymic properties of the three 'oxacillinases' is presented. With the OXA2 enzyme, 'burst' kinetics, implying branched pathways, seemed to prevail with many substrates.

INTRODUCTION

Two groups of β-lactamases can be distinguished on the basis of their catalytic mechanisms. Class-B enzymes are metalloproteins containing at least one Zn^{2+} ion per active subunit (Ambler, 1980). In contrast, β-lactamases of classes A, C and D are active-site serine enzymes (Jaurin and Grunström, 1981; Mossakowska et al., 1989). These three classes differ in their primary structures. Members of classes A and C have been widely studied and some high-resolution three-dimensional structures have been obtained (Kelly et al., 1986; Samraoui et al., 1986; Oefner et al., 1990; Knox and Moews, 1991; Herzberg, 1991). At the present time, five enzymes clearly belong to class D: OXA1, OXA2, OXA5, LCR1 and PSE2. The genes have been sequenced (Dale et al., 1985; Ouellette et al., 1987; Huovinen et al., 1988; Couture et al., 1992), and the deduced primary structures do not exhibit statistically significant similarities to those of the enzymes of classes A and C (Joris et al., 1988). Nonetheless, a close analysis (Joris et al., 1991) shows that some of the motifs bordering the active sites of classes A and C enzymes and of the R61 DD-peptidase can be found in similar positions in class-D β-lactamases. In consequence, it seems safe to assume that Ser-70 [numbering of Joris et al. (1991)] is the active serine of class-D enzymes. The class-D enzymes exhibit original substrate profiles, oxacillin being the most sensitive to hydrolysis, in contrast with the situation with many class-A enzymes (Matagne et al., 1990) and, even more so, with all class-C enzymes (Galleni and Frère, 1988; Galleni et al., 1988). If these observations can be extrapolated, it is likely that the OXA3, OXA4, OXA6 and OXA7 enzymes also belong to class D (Medeiros et al., 1985). Immunological cross-reactions have, for instance, been observed with antibodies raised against the OXA2 and OXA3 proteins (Holland and Dale, 1985).

The present investigation was initiated to obtain detailed data about the chemical and catalytic properties of class-D β-lactamases. Three of the recognized members of the class have been studied.

MATERIALS AND METHODS

Bacterial strains and plasmids

Plasmid R46 (52 kb) was the source of the OXA2 gene (Anderson and Datta, 1965), plasmid pMON234 (5.05 kb) was the source of the PSE2 gene (Huovinen et al., 1988) and plasmid pBGS18+ (4.4 kb) was used as a vector (Spratt et al., 1986). *Escherichia coli* JM105 [*endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*)] was used for the production of the OXA2 β-lactamase, *E. coli* HB101 (*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) for the production of the PSE2 β-lactamase and *E. coli* HB101 (pMON301) (Levesque et al., 1987) for the production of the OXA1 β-lactamase.

Antibiotics

Ampicillin and oxacilin were from Bristol Benelux S.A. (Brussels, Belgium), 6-aminopenicillanic acid, carbenicillin, cloxacillin, methicillin, flucloxacillin and dicloxacillin were from Beecham Research Laboratories (Brentford, Middx., U.K.), benzylpenicillin was from Rhône-Poulenc (Paris, France), cefamandole, cefazolin, cephaloglycin, cephaloridine and cephalosporin C were from Eli Lilly and Co. (Indianapolis, IN, U.S.A), 6β-iodopenicillanic acid was from Pfizer Central Research (Sandwich, Kent, U.K.), ceftazidime was from Glaxo Group Research (Greenford, Middx, U.K.) and cefotaxime was from Hoechst-Roussel (Romainville, France). All these compounds were kindly given by the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hants, U.K.) and 7-aminocephalosporanic acid from Janssen Pharmaceutica (Beerse, Belgium). 6β-Iodo[^{3}H]penicillanic acid (2.1 mCi/mmol) was the sample described previously (De Meester et al., 1985). Kanamycin was purchased from Boehringer (Mannheim, Germany).

Proteins

Tos-Phe-CH_2Cl ('TPCK')-treated trypsin was from Millipore Corp. (Freehold, NJ, U.S.A.), BSA and ovalbumin were from

Abbreviations used: BH medium, brain/heart medium; TB, Terrific broth.

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Sigma Chemical Co. (St. Louis, Mo, U.S.A.) and lysozyme was from Belovo (Bastogne, Belgium).

Protein concentrations were estimated by measuring the A_{280} of the solutions.

Growth media

Brain/heart (BH) medium contained, per litre, 37 g of brain/heart infusion (bioMérieux, Charbonnières-les-Bains, France). 'Terrific broth' medium (TB) contained, per litre, 12 g of Bactotryptone (Difco), 24 g of Bacto yeast extract (bio-Mérieux) 4 ml of glycerol and 100 ml of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 buffer.

Chromatography

Amberlite CG50 was from Serva (Heidelberg, Germany), Sephadex G-25, Sephadex G-75, Sephadex 75 HR 10/30, Sephadex G-100, Mono S HR 5/5, Q Sepharose HiLoad 26/10 and PepRPC HR 5/5 were from Pharmacia (Uppsala, Sweden). The agarose–phenylboronic acid (type B) column was prepared as described by Cartwright and Waley (1984).

Spectrophotometers

All the spectrophotometric measurements were performed with the help of a Uvikon 860 (Kontron instruments) spectrophotometer interfaced to a Copam+ PC 88C microcomputer.

Gas-phase sequenator

The amino acid sequence of the active-site peptide was determined with an Applied Biosystems 470-A gas-phase sequenator.

Genetic techniques

Standard DNA recombinant techniques were used (Maniatis et al., 1982).

Kinetic measurements

Usually, a complete time course of the hydrolysis of the substrate was recorded at 482 nm for nitrocefin, at 260 nm for the other cephalosporins, at 240 nm for carbenicillin, at 260 nm for oxacillin, cloxacillin, dicloxacillin and methicillin, at 272 nm for flucloxacillin, and at 235 nm for the other penicillins. The interaction between 6β-iodopenicillanic acid and the OXA2 β-lactamase was studied by using the reporter substrate method (De Meester et al., 1987; Galleni and Frère, 1988). When biphasic hydrolysis time courses were observed, the values of the initial rates (v_0), the steady-state rates (v_{ss}), and the partial inactivation and re-activation rate constants (k_i and k_r respectively) were determined with the help of the microcomputer as described by De Meester et al. (1987).

Dilutions of the enzymes below a concentration of 0.1 mg/ml were performed with buffer solutions containing 0.1 mg of BSA/ml.

RESULTS

Subcloning of the OXA2 gene from the R46 plasmid in the pBGS18$^+$ multicopy vector

In order to increase the production of the OXA2 β-lactamase, the OXA2 gene was subcloned by inserting the 2.5 kb *Pst*I 50860–*Bam*HI 1160 DNA fragment of R46 into the *Bam*HI–*Pst*I site of the multiple-cloning region of pBGS18$^+$. The ligation mixture was used to transform *E. coli* JM105. Ampicillin-resistant and kanamycin-resistant transformants were selected. The recombinant plasmid pDML303 was isolated, purified and its restriction map established (Figure 1).

The levels of β-lactamase production by *E. coli* JM105 transformed by R46 or pDML303 are compared in Table 1.

Fusion of the pMON234 plasmid harbouring the PSE2 gene with the pBGS18$^+$ vector

As for OXA2, various subcloning experiments were attempted in pBGS18$^+$. No ampicillin- and kanamycin-resistant transformants were obtained when the transformation was made with a ligation mixture in which the PSE2 gene was on a short fragment (i.e. the 1350 pb *Bam*HI–*Sph*I or the 1550 pb *Bam*HI–*Sst*I fragments). However, each of the two plasmids contains only one *Eco*RI site. Ligation of the plasmids after digestion by *Eco*RI yielded the recombinant plasmid pBGS18$^+$::pMON234 (Figure 1). After transformation with pBGS18$^+$::pMON234, *E. coli* HB101 overproduced the PSE2 β-lactamase (Table 1).

Production and purification of the OXA2 β-lactamase

Twenty 1-litre conical flasks each containing 500 ml of BH medium were inoculated with 10 ml of a 16 h preculture in the same medium and stirred overnight at 37 °C. The cells were separated by centrifugation and submitted to three rounds of sonication (2 × 3 min). The combined supernatants were centrifuged for 30 min at 39000 *g*. A total β-lactamase activity of 27600 μmol/min was measured with benzylpenicillin as substrate.

The isoelectric pH of the OXA2 enzyme being 8.0 (±0.5), a batch adsorption on a CG50 cation exchanger was performed after adjusting the pH to 6.0 with diluted H_3PO_4. Two successive desorptions with 0.1 M Tris/H_3PO_4 buffer, pH 8.6, containing 0.25 M K_2SO_4 allowed the recovery of 71% and 10% of the initial β-lactamase activity respectively.

The last step of the purification was affinity chromatography on an agarose–phenylboronic acid (type B) column. The OXA2 enzyme was only retarded on washing with the Tris/H_3PO_4/K_2SO_4, buffer. Three successive passages were necessary to obtain the pure enzyme. Table 2 summarizes the different steps of the purification.

Production and purification of the PSE2 β-lactamase (Table 3)

An initial test of production was made in 100 ml of TB medium. A linear increase in β-lactamase activity was observed after the end of the exponential phase. The activity after 45 h of culture at 37 °C was approximately ten times higher than after 12 h.

The bacteria were grown for 2 days at 37 °C in a 20-litre fermenter containing 15 litres of TB medium. After centrifugation, the cells were suspended in 100 mM Tris adjusted to pH 9.4 with EDTA. Four rapid freeze–thaw cycles were performed to extract the PSE2 β-lactamase from the periplasm.

A batch adsorption–desorption on a CG50 cation exchanger was also the first purification step for this enzyme. The isoelectric pH of the enzyme is 6.1 and thus adsorption was completed by adjusting the mixture to pH 4.6 with 5 M H_3PO_4. Two successive desorptions in 50 mM sodium phosphate buffer, pH 7.6, allowed the recovery of 46% and 14% of the initial β-lactamase activity respectively.

The sample was filtered at 4 °C through a Sephadex G-100 column (80 cm × 4.0 cm) in 50 mM sodium phosphate buffer,

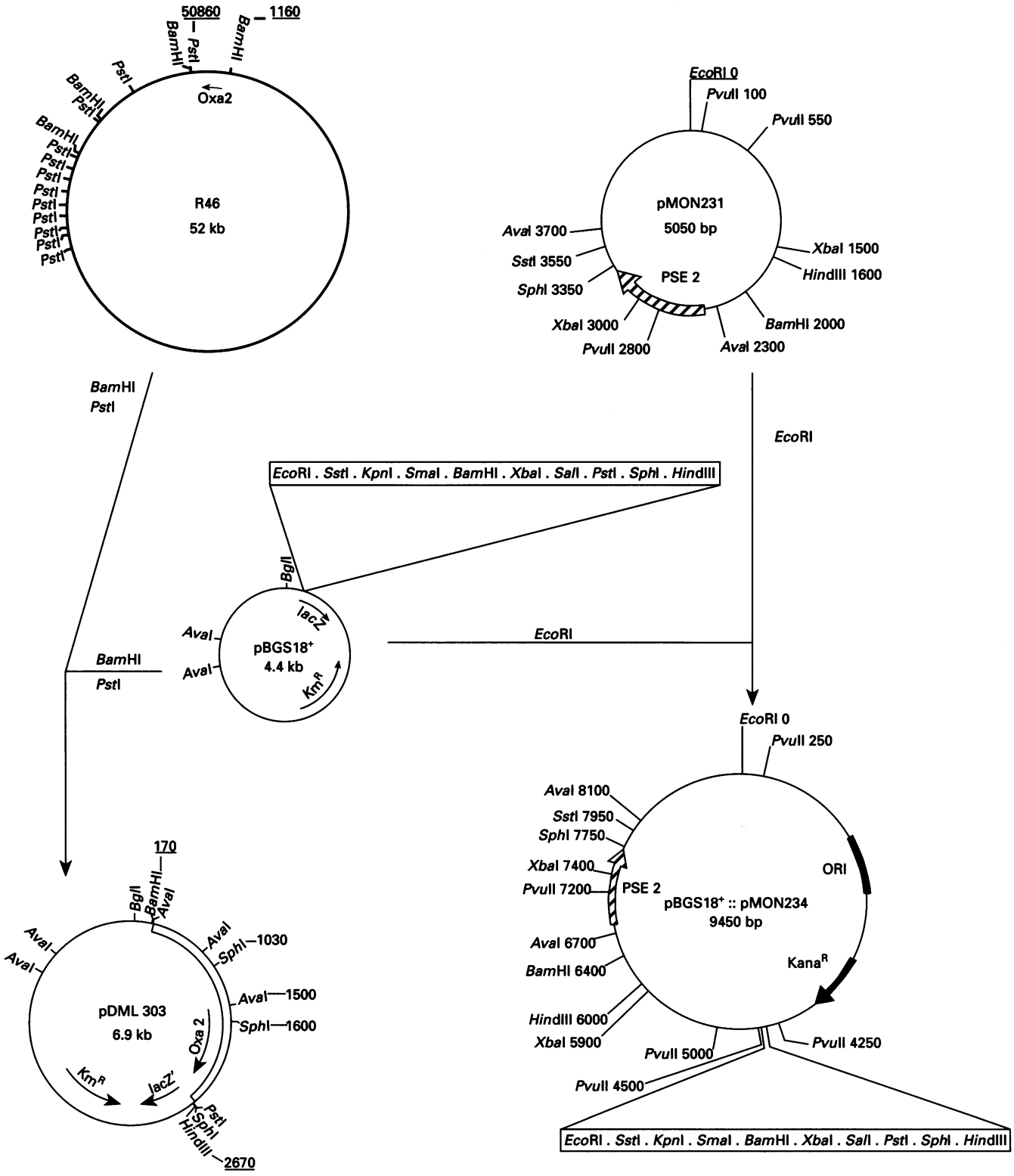


Figure 1 Construction of the plasmids pDML303 and pBGS18⁺::pMON234

pH 7.6, at a flow rate of 30 ml/h. The β-lactamase was eluted at a volume of 320 ml. Fractions containing β-lactamase activity were pooled and concentrated.

A Q-Sepharose Hiload column coupled with an f.p.l.c. apparatus was then used for the last step of the purification: buffers A and B were 50 mM sodium phosphate, pH 7.6, and 50 mM sodium phosphate, pH 7.6, containing 0.5 M K_2SO_4 respectively; the gradient went from 0 to 100 % of buffer B in 500 ml; the flow rate was 5 ml/min. The β-lactamase was eluted at a volume of 100 ml corresponding to 20 % of buffer B.

Table 1 Levels of production of the OXA2 and PSE2 β-lactamases

The values are expressed in μmol of substrate hydrolysed/min per litre of culture. They represent the means of five measurements ± S.D.

Strains	Enzyme produced	Substrate	Activity (μmol/min per l)
E. coli (R46)	OXA2	Benzylpenicillin (500 μM)	160 (±15)
E. coli (pDML303)	OXA2	Benzylpenicillin (500 μM)	640 (±40)
E. coli (pMON234)	PSE2	Nitrocefin (100 μM)	10 (±1)
E. coli (pBGS18$^+$::pMON234)	PSE2	Nitrocefin (100 μM)	360 (±20)

Table 2 Purification of the OXA2 β-lactamase

The values in parentheses give the yield (%) of each step. The activity is expressed as μmol of benzylpenicillin hydrolysed/min per mg of protein.

		Total activity (μmol/min)	Total proteins (mg)	Specific activity (μmol/min per mg of protein)	Purification factor	Recovered activity (%)
Crude extract		27600	25000	1.1	–	100
CG50		22400	2000	11	10	81
Phenylboronate	1	19300	650	30	27	70 (86)
	2	15250	110	140	127	55 (79)
	3	14000	56	250	227	51 (92)

Table 3 Purification of the PSE2 β-lactamase

The activity is expressed as μmol of oxacillin hydrolysed/min per mg of protein. The yield (%) of each step is given in parentheses.

	Total activity (μmol/min)	Total proteins (mg)	Specific activity (μmol/min per mg of protein)	Purification factor	Recovered activity (%)
Crude extract	17000	48000	0.35	–	100
CG50	10200	6700	1.5	4.3	60
G100	8300	2500	3.3	9.5	49 (81)
Q Sepharose HL	6200	150	41	117	36 (75)

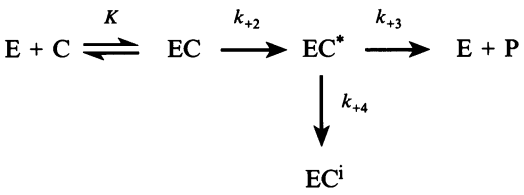
Production and purification of the OXA1 β-lactamase (Table 4)

A 1-litre conical flask containing 500 ml of TB medium was inoculated with 10 ml of a 16 h preculture in the same medium and stirred overnight at 37 °C. The cells were separated by centrifugation, resuspended in 100 mM Tris adjusted to pH 9.5 with EDTA and submitted to two freeze–thaw cycles. The solution was filtered at 4 °C through a Sephadex G-100 column (80 cm × 4.0 cm) in 10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 40 ml/h. The β-lactamase was eluted at a volume of 440 ml. Active fractions were pooled, concentrated and dialysed against 50 mM potassium acetate, pH 4.5.

Table 4 Purification of the OXA1 β-lactamase

For details, see the legend to Table 3.

	Total activity (μmol/min)	Total proteins (mg)	Specific activity (μmol/min per mg of protein)	Purification factor	Recovered activity (%)
Crude extract	60	150	0.40	–	100
Sephadex G-100	52	40	1.3	3.3	87
Mono S	36	2.7	13.3	33	60 (69)



Scheme 1 General model for the interaction of β-lactamase (E) with 6β-iodopenicillanate (C)

EC* is the acylenzyme and EC^i the rearranged adduct containing the dihydrothiazine chromophore.

The sample was then submitted to chromatography on a Mono S column coupled to an f.p.l.c. apparatus. Buffers A and B were 50 mM potassium acetate adjusted to pH 4.5 with concentrated acetic acid and the same buffer containing 0.5 M K_2SO_4 respectively; the flow rate was 0.5 ml/min and the gradient went from 0 to 100 % of buffer B in 25 ml. The β-lactamase was eluted at a volume of 17 ml corresponding to 66 % of buffer B.

Interaction of the OXA2 β-lactamases with 6-β-iodopenicillanic acid

Titration of the OXA2 β-lactamase with 6β-iodopenicillanic acid

A 'branched-pathway' mechanism (Scheme 1) seems to prevail in the interaction of this β-lactam with several class-A β-lactamases where the acylenzyme can rearrange into an irreversibly inactivated species or undergo normal hydrolysis (De Meester et al., 1986). The hydrolysis product and the rearranged inactivator moiety molecule bound to the active site of the enzyme both contain a dihydrothiazine chromophore. The adsorption maxima are at 305 nm ($\epsilon = 8200\ M^{-1}\cdot cm^{-1}$) and at 325 nm ($\epsilon = 12500\ M^{-1}\cdot cm^{-1}$) for the former and the latter respectively (Frère et al., 1982).

Titration of the OXA2 β-lactamase with 6β-iodopenicillanate showed that this reagent inactivated the enzyme without apparent turnover ($k_{+4} \gg k_{+3}$). The inactivator/enzyme ratio necessary to obtain total inactivation was 1:1(±5%). Indeed, at the end of the titration, the absorption spectrum exhibited a clear maximum at 325 nm (Figure 2).

The enzyme concentration determined on the basis of the titration end point (27.6 μM) was in good agreement with that deduced from the absorbance value at 325 nm (27.2 μM). From the known molecular weight and the tryptophan and tyrosine content of the enzyme, the theoretical A_{280} (1 %) value (Cantor and Schimmel, 1980) could be computed (21.9), yielding a

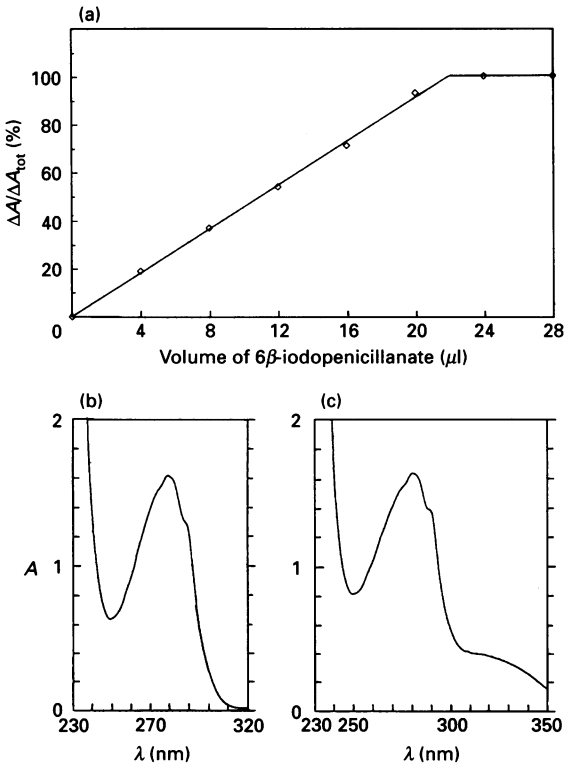


Figure 2 Interaction of the OXA2 β-lactamase with 6β-iodopenicillanic acid

(**a**) Titration of the OXA2 β-lactamase by 6β-iodopenicillanic acid. Identical samples of inactivator (2.4 nmol in 4 μl) were added to 500 μl of the enzyme solution until no further increase in A_{325} was observed. The end point of the titration corresponded to 23 μl (13.8 nmol) of 6β-iodopenicillanic acid. ($\Delta A = A_{325}$ after each addition of inactivator $-A_{325}$ of the solution without inactivator; $\Delta A_{tot.} = A_{325}$ at the titration end point $-A_{325}$ of the solution without inactivator.) (**b**) and (**c**) Absorption spectra of the OXA2 β-lactamase before (**b**) and after (**c**) inactivation by 6β-iodopenicillanic acid.

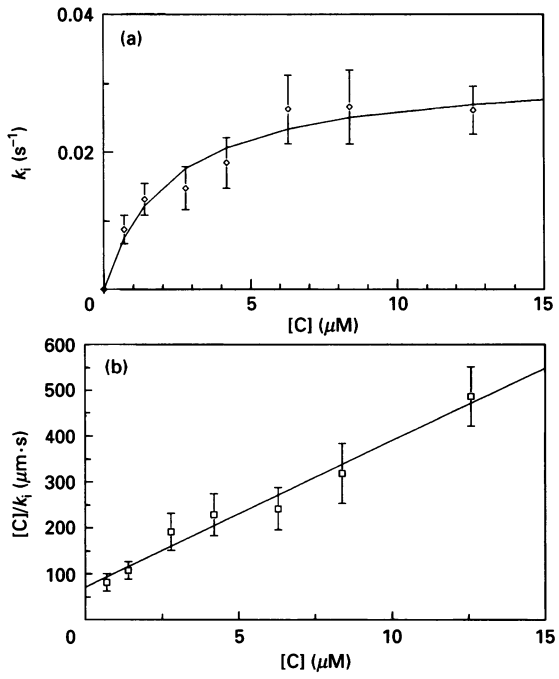


Figure 3 Inactivation of the OXA2 β-lactamase with 6β-iodopenicillanic acid

(**a**) Pseudo-first-order inactivation rate constant (k_i) as a function of the inactivator concentration (C). Each point is the mean ± S.D. of five measurements. (**b**) Plot of $[C]/k_i$ as a function of [C]: $r = 0.985$.

25.4 μM concentration of enzyme in the titration experiment, thus in good agreement with the values determined above.

Kinetic measurements

The determination of constants k_{+2} and K was made with oxacillin (500 μM) as a reporter substrate. Pseudo-first-order inactivation rate constants (k_i) were measured for inactivator concentrations ranging from 0.7 μM to 12.6 μM (Figure 3). After correction for the protection by the reporter substrate, the constants k_{+2} and K were calculated with the help of eqn. (1) (Frère et al., 1982):

$$\frac{[I]}{k_i} = \frac{K\left(\frac{K_m^S + [S]}{K_m^S}\right)}{k_{+2}} + \frac{1}{k_{+2}}[I] \qquad (1)$$

where K_m^S and [S] are respectively the K_m for and the concentration of the reporter substrate. The k_{+2}, K and k_{+2}/K values were respectively 3.2 (± 0.2) $\times 10^{-2}$ s^{-1}, 0.95 (± 0.20) μM and 34000 (± 7000) $M^{-1} \cdot s^{-1}$. The errors were those obtained for the parameters after a linear regression with the Enzfitter program (Leatherbarrow, 1987).

The value of k_{+2} was verified by directly following the absorbance at 325 nm. Under these conditions ([6β-iodopenicillanate] = 100 μM; [OXA2] = 1–10 μM), k_i was equal to k_{+2} for which a value of 2.5 (± 0.7) $\times 10^{-2}$ s^{-1} was found, in good agreement with that determined above.

Identification of the active-site serine of the OXA2 β-lactamase

Sequence alignments indicated Ser-70 as the probable active-site serine residue. In order to verify this assumption, the enzyme was labelled by adding 95 nmol of 6β-iodo[^{3}H]penicillanate to a stoichiometric amount of enzyme in a total volume of 3.5 ml. After 10 min at 30 °C, the sample was freeze-dried, and the solid residue redissolved in 500 μl of 8 M urea. The solution was incubated for 6 h at 37 °C and the urea concentration decreased to 4 M by addition of 500 μl of water. Trypsin digestion was realized in two steps: after addition of 250 μg of trypsin, the sample was incubated for 12 h at 37 °C and a further 3 h digestion was performed after a second addition of the same amount of the same protease at the same temperature.

The digest was filtered through a Sephadex G-25 column (120 cm × 1 cm) in 10 mM NH_4HCO_3. The elution profile showed four radioactive peaks; fractions corresponding to the smallest labelled peptides (18 nmol) were pooled and freeze-dried. The powder was redissolved in 500 μl of 1% trifluoroacetic acid in water and further purified with the help of f.p.l.c. apparatus equipped with a PEP-RPC HR(5/5) column. The solvents were 0.1% (v/v) trifluoroacetic acid in water (A) and in acetonitrile (B). The flow rate was 0.7 ml/min and the gradient went from 0 to 60% (v/v) of solvent B over 42.5 ml. The labelled peptide was eluted at a volume corresponding to 28% of solvent B.

The results of gas-phase sequencing showed the presence of two peptides. Some residues were difficult to identify with certainty and it has been noted before that the yield obtained for

Table 5 Active-site peptide sequencing

	32	39	66	70	73
Sequence (Dale, 1985)	F F S E F Q A K		Y S P A S*T F K		
Fraction 49: peptide 1	F F S E – Q A K				
peptide 2			Y – P A – – F K		

* Residue not identified with certainty.

Table 6 Apparent molecular masses of the OXA2, PSE2 and OXA1 β-lactamases as determined on Sephadex 75 HR 10/30, and $K_{av.}$ values

The linear-regression analysis [log (molecular mass) = $f(K_{av.})$] was performed with BSA, ovalbumin and lysozyme ($r = -0.988$).

Proteins	Molecular mass (kDa)	$K_{av.}$
BSA	66	0.118 ± 0.001
Ovalbumin	43	0.183 ± 0.001
Lysozyme	14.4	0.602 ± 0.002
OXA2	50.6 ± 0.9	0.171 ± 0.002
PSE2	35.3 ± 0.6	0.293 ± 0.003
OXA1	24.6 ± 1	0.415 ± 0.015

the dihydrothiazine-labelled serine was generally poor on gas-phase sequencing (Amicosante et al., 1988). Although the sequences were incomplete, they could be replaced in the amino acid sequence deduced from the known OXA2 nucleotide sequence (Dale et al., 1985), and, in particular, the well-identified residues of peptide 2 allowed an unambiguous positioning in the sequence. As predicted by the sequence alignments, it could be concluded that Ser-70 was the active-site serine residue (Table 5).

Behaviour of the OXA1, OXA2 and PSE2 β-lactamases on Sephadex 75

The determination of the apparent molecular mass of the three class-D β-lactamases was performed on a Sephadex 75 HR 10/30 column coupled to an f.p.l.c. apparatus. The K_{av} values and the corresponding molecular masses are given in Table 6. The three class-D β-lactamases were characterized by quite different apparent molecular masses. Those of OXA2 and PSE2 were higher than those deduced from their primary structures; that of OXA1 was lower than 30 kDa.

The high apparent molecular mass of the OXA2 β-lactamase on Sephadex 75 HR was not due to non-specific interactions between the protein and the matrix since these would increase the $K_{av.}$, leading to a lower apparent molecular mass. It has been suggested that the OXA2 and OXA3 enzymes could exist as dimeric proteins (Dale and Smith, 1976), which would explain the high apparent molecular mass of the former. In consequence, we analysed the behaviour of the OXA2 enzyme under different conditions which might influence the equilibrium between dimer and monomer.

The $K_{av.}$ value on the Sephadex 75 HR column was not influenced by pH variations (from 4 to 10), high ionic strength (0.5 M Na_2SO_4) or the presence of 200 mM borate (a reversible inhibitor), 1 M $NaClO_4$ (a dissociating agent) or 1 M guanidinium chloride. Preincubation of the enzymes with

Table 7 Substrate profiles of OXA2, OXA1 and PSE2 β-lactamases: penicillins

v_0 and v_{ss} are expressed as percentages of the v_{ss} of benzylpenicillin arbitrarily taken as 100%. The k_i value characterizes the rate of transition to the final steady state. It corresponds to an increase in activity for methicillin (lag) and to a decrease in all other cases. Abbreviations: 6-APA, 6-aminopenicillanic acid; ND, not determined; +, burst; 0, no burst; ±, very small burst; /, not relevant since no burst was observed.

Substrate	OXA2	OXA1	PSE2
6-APA (500 μM)	+	0	0
v_0	170	70	16
v_{ss}	98	/	/
v_0/v_{ss}	1.7	/	/
k_i (s^{-1})	28×10^{-3}	/	/
Benzylpenicillin (500 μM)	+	0	0
v_0	140	100	100
v_{ss}	100	/	/
v_0/v_{ss}	1.4	/	/
k_i (s^{-1})	35×10^{-3}	/	/
Carbenicillin (500 μM)	+	+	+
v_0	80	110	120
v_{ss}	2.3	63	28
v_0/v_{ss}	35	1.7	4.3
k_i (s^{-1})	77×10^{-3}	10×10^{-3}	27×10^{-3}
Methicillin (1 mM)	+	lag	lag
v_0	45	155	75
v_{ss}	14	390	230
v_0/v_{ss}	3.2	0.4	0.3
k_i (s^{-1})	14×10^{-3}	47×10^{-3}	61×10^{-3}
Oxacillin (1 mM)	0	±	±
v_0	710	260	500
v_{ss}	/	180	430
v_0/v_{ss}	/	1.4	1.2
k_i (s^{-1})	/	7.6×10^{-3}	15×10^{-3}
Cloxacillin (1 mM)	+	+	+
v_0	220	250	1430
v_{ss}	48	75	230
v_0/v_{ss}	4.6	3.3	6.2
k_i (s^{-1})	42×10^{-3}	6.9×10^{-3}	22×10^{-3}
Dicloxacillin (1 mM)	+	+	+
v_0	120	280	1460
v_{ss}	21	55	155
v_0/v_{ss}	5.7	5.1	9.4
k_i (s^{-1})	22×10^{-3}	14×10^{-3}	24×10^{-3}
Flucloxacillin (1 mM)	0	+	+
v_0	Competitive	110	1
v_{ss}	inhibitor	1.5	0.2
v_0/v_{ss}		73	5
k_i (s^{-1})		20×10^{-3}	ND

cephaloridine, a substrate exhibiting burst kinetics (see below), also failed to modify the enzyme behaviour.

Affinity chromatography on an agarose–phenylboronate column of the OXA2 β-lactamase partially inactivated by 6β-iodopenicillanate

The dimeric nature of the OXA2 β-lactamase did not seem to be confirmed by the gel-filtration results. The existence of a rapid equilibrium between monomeric and dimeric forms of the enzyme was further investigated by chemical derivatization experiments coupled with affinity chromatography on an agarose–phenylboronate column.

A sample of the OXA2 β-lactamase was 50% inactivated by 6β-iodopenicillanate so that the ratio of adduct to intact enzyme was 1. The mixture was applied to the affinity column which was eluted at 20 °C with 0.1 M Tris/phosphate, pH 8.6, containing

Table 8 Substate profiles of OXA2, OXA1 and PSE2 β-lactamases: cephalosporins

For the legend, see Table 7. 7-ACA, 7-aminocephalosporanic acid. v_0 and v_{ss} are expressed as percentages of the v_{ss} value of benzylpenicillin.

Substrate	OXA2	OXA1	PSE2
7-ACA (200 μM)	+	0	0
v_0	0.67	1.1	0.1
v_{ss}	0.13	slow and	/
v_0/v_{ss}	5.0	total	/
k_i (s^{-1})	19×10^{-3}	inactivation	/
Cephaloglycin (200 μM)	+	0	0
v_0	3.1	3.5	3.6
v_{ss}	0.5	/	/
v_0/v_{ss}	6.2	/	/
k_i (s^{-1})	19×10^{-3}	/	/
Cephalosporin C(300 μM)	+	±	0
v_0	46	2.2	3.0
v_{ss}	2.4	1.7	/
v_0/v_{ss}	19	1.3	/
k_i (s^{-1})	29×10^{-3}	12×10^{-3}	/
Cephaloridine (150 μM)	+	0	±
v_0	45	34	8.2
v_{ss}	1.4	/	6.8
v_0/v_{ss}	32	/	1.2
k_i (s^{-1})	42×10^{-3}	/	19×10^{-3}
Ceftazidime (100 μM)	+	0	0
v_0	0.12	2.6	0.02
v_{ss}	0.02	Slow and	/
v_0/v_{ss}	6	total	/
k_i (s^{-1})	41×10^{-3}	inactivation	/
Cefazolin (150 μM)	+	0	0
v_0	16	13	3.1
v_{ss}	3.6	/	/
v_0/v_{ss}	4.4	/	/
k_i (s^{-1})	25×10^{-3}	/	/
Cefamandole (200 μM)	+	0	0
v_0	8.4	9.5	10
v_{ss}	6.5	/	/
v_0/v_{ss}	1.3	/	/
k_i (s^{-1})	4.5×10^{-3}	/	/
Nitrocefin (150 μM)	+	0	0
v_0	80	215	165
v_{ss}	Complex	/	/
v_0/v_{ss}	kinetics	/	/
k_i (s^{-1})		/	/

0.25 M K_2SO_4; the flow rate was 2.5 ml/min. The elution profile clearly showed the presence of two peaks. The first one, centred on the dead volume, corresponded to β-lactamase labelled by the inactivator; its absorbance spectrum exhibited a secondary maximum at 325 nm. The second peak contained non-labelled enzyme interacting with the boronate groups. The same result was obtained with adduct/free enzyme ratios of 1:3 and 3:1; the peak areas determined at 280 nm were proportional to these ratios.

This experiment invalidated the hypothesis of a rapid equilibrium between monomeric and dimeric forms of enzyme. In the presence of such an equilibrium, only one peak should have been observed, at an elution volume intermediate between those of the non-retarded and retarded forms of enzyme.

Heterogeneity of the OXA2 β-lactamase on ion exchangers

Matthew and Hedges (1976) and Matthew et al. (1979) clearly showed the existence of at least three bands on isoelectric focusing of an OXA2 preparation. In order to determine if these various forms of enzyme had similar kinetic properties, the following experiment was performed. A sample of OXA2 β-lactamase (50 mg) was loaded on to a Q Sepharose Hiload 26/10 column with 50 mM sodium phosphate, pH 7.0, at a flow rate of 5 ml/min. During the washing of the column with the same buffer, four distinct peaks were separated. After 330 ml, the same buffer containing 0.5 M K_2SO_4 was used and two other peaks appeared within the next 100 ml. The fractions centred on each of the peaks were pooled, concentrated and their kinetic properties studied. The hydrolysis of cephaloridine by the OXA2 β-lactamase being biphasic, an initial rate/steady-state ratio (v_0/v_{ss}) and a partial inactivation rate constant (k_i) could be measured (see below). These different parameters were the same for all the enzyme forms. Moreover, the proteins in the six separate fractions exhibited similar mobilities on SDS/PAGE.

Substrate profile of OXA1, OXA2 and PSE2 β-lactamases

Tables 7 and 8 give the relative rates of hydrolysis of several penicillins and cephalosporins respectively (benzylpenicillin being the reference) and, when a biphasic hydrolysis was observed, the v_0/v_{ss} ratios and the first-order rate constant k_i characterizing the rate of transition to the final steady state. As could be predicted from the data available in the literature, the three enzymes consistently hydrolysed oxacillin and cloxacillin faster than benzylpenicillin, justifying their appellation as 'oxacillinases'. However, surprisingly, in most cases, the hydrolysis time courses did not correspond to the simple Henri–Michaelis model. 'Bursts' were often observed and 'lags' in the hydrolysis of methicillin by OXA1 and PSE2. In all these experiments, the enzyme concentration (0.04–0.1 μM) was much lower than that of the substrate (0.1–1 mM) and the size of the burst was significantly larger than the enzyme concentration, thus indicating that the burst was not due to acylenzyme accumulation but more probably to the occurrence of a branched pathway.

The v_0/v_{ss} ratios increased with the number of Cl atoms on the isoxazolyl side chain but the replacement of the second Cl with F produced specific results depending on the enzyme.

Cephalosporins, with the possible exception of nitrocefin, were generally poorer substrates than penicillins. With these substrates, the behaviour of OXA2 was clearly different from those of the two other enzymes. Indeed, OXA2 did not hydrolyse any of the cephalosporins according to simple Henri–Michaelis kinetics whereas OXA1 and PSE2 generally obeyed this simple model.

DISCUSSION

During this study, we have purified three class-D β-lactamases to protein homogeneity. Our protocol of purification of the OXA2 and OXA1 enzymes gave higher specific activities and better yields than those previously published (Yamagishi *et al.*, 1969; Dale and Smith, 1971; Monaghan *et al.*, 1982; Holland and Dale, 1984). Philippon et al. (1983) purified the PSE2 β-lactamase 56-fold with a 26% yield but did not indicate the specific activity of the pure enzyme.

Surprisingly, despite the apparent lack of affinity for 3-aminophenylboronate, the OXA2 β-lactamase was retarded on the affinity column. This phenomenon seemed to be specific since the inactivation of the enzyme by 6β-iodopenicillanate appeared to suppress the interaction. One might argue that the observed results were due to an ion-exchange phenomenon and that the additional negative charge supplied by the inactivator moiety was sufficient to modify the behaviour of the enzyme, but that

seems unlikely since the chromatography was performed in the presence of 0.5 M K_2SO_4.

The interaction of the OXA2 β-lactamase with 6β-iodopenicillanate followed a simple linear pathway and the k_{+2}/K value was in the range of those observed with class-A β-lactamases. Moreover, the K value was strikingly low.

Isolation of an active-site peptide after labelling with 6β-iodopenicillanate allowed the identification of Ser-70 as the active-site serine residue. This was in agreement with the tentative identification based on the gene sequence (Dale et al., 1985). It could be argued that peptide 1 also contained a serine residue, but Ser-34 is not conserved in other class-D enzymes, which excludes it as the potential active-site serine (Joris et al., 1991). Moreover, in the analogous class-A β-lactamases (Joris et al., 1991), the corresponding peptide is part of an α-helix in the α/β domain, far away from the active site.

In agreement with the results of Dale (1971) and Foster (1983), the behaviour of the OXA2 β-lactamase during molecular-sieve chromatography indicated an M_r value twice as large as that of OXA1 and considerably larger than that deduced from the gene sequence. Since covalent linking of two identical subunits could be rejected on the basis of the normal behaviour of the enzyme on SDS/PAGE and the absence of cysteine residues in the sequence, we explored the possibility of a rapid equilibrium between a monomer and a dimer. No conditions were found that could increase the elution volume of the enzyme. Moreover, when 6β-iodopenicillanate-inactivated enzyme was mixed with the intact protein, no subunit interchange leading to the formation of hybrid dimers (or polymers) could be detected. In conclusion, the causes of the apparently larger M_r value observed for the OXA2 enzyme during molecular-sieve chromatography remain a mystery, since on the basis of the structural analogies with the class-A β-lactamases, one would not expect the OXA2 protein to present an especially elongated shape.

As observed with many other β-lactamases (Matagne *et al.*, 1991), several different forms of the OXA2 enzyme could be separated by ion-exchange chromatography. The M_r values of all these forms, determined by SDS/PAGE, were similar and it was safe to conclude that they originated from slightly 'ragged' N-termini or deamidation of particularly fragile asparagine residue(s). Moreover, the catalytic properties of the various forms appeared to be identical.

A first overview of the catalytic properties of the three enzymes has been realized. As expected, the hydrolysis of oxacillin was catalysed more efficiently than that of benzylpenicillin. Moreover, the most unexpected result was the finding that, at least with the OXA2 enzyme, 'burst' kinetics seemed to prevail with many substrates. In fact, this phenomenon perfectly explained the wide dispersion of the $V_{max.}$ or initial-rate values found in the literature. Indeed, we observed an apparent correlation between that dispersion and the size of the 'burst'. The two other enzymes more consistently produced 'bursts' with substrates that are known to induce that type of behaviour, such as the isoxazolyl penicillins. These phenomena, which imply the existence of a branched pathway, are at present under detailed investigation.

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